1 **Intestinal immune responses of Jian carp against *Aeromonas hydrophila***
2 **depressed by choline deficiency: varied change patterns of mRNA levels of**
3 **cytokines, tight junction proteins and related signaling molecules among three**
4 **intestinal segments**

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6 Pei Wu ^{1,2,3}, Yang Liu ^{1,2,3}, Wei-Dan Jiang ^{1,2,3}, Jun Jiang ^{2,3}, Yong-An Zhang ⁴,
7 Xiao-Qiu Zhou ^{1,2,3}*, Lin Feng ^{1,2,3}*

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9
10 ¹*Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, China*

11 ²*Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province,*
12 *Sichuan Agricultural University, Chengdu, 611130, China*

13 ³*Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of*
14 *Education, Sichuan Agricultural University, Chengdu, 611130, China*

15 ⁴*Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China*
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17 **Running title: Choline ameliorated intestinal inflammation of fish**

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19 * Corresponding authors. Tel.: +86-835-2885157; Fax: +86-835-2885968.

20 *E-mail address:* zhouxq@sicau.edu.cn (X.-Q. Zhou); fenglin@sicau.edu.cn (L. Feng).

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Abstract

This study aimed to investigate the effects of choline deficiency on intestinal inflammation of fish after *Aeromonas hydrophila* infection and the potential molecular mechanisms. Juvenile Jian carp (*Cyprinus carpio* var. Jian) were fed two diets containing choline at 165 (deficient group) and 607mg/kg diet respectively for 65 days. Choline deficiency decreased intestinal lysozyme activity, C3 and IgM contents, increased acid phosphatase activity, downregulated mRNA levels of antimicrobial peptides [liver-expressed antimicrobial peptide (LEAP) 2A, LEAP-2B, hepcidin and defensin], cytokines [interleukin (IL) 6a, tumor necrosis factor α (TNF- α), interferon γ 2b (IFN- γ 2b), IL-6b and transforming growth factor β 2 (TGF- β 2) only in proximal intestine, IL-10 in mid and distal intestine], immune-related signaling molecules [Toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), nuclear factor kappa B (NF- κ B), inhibitor of NF- κ B (I κ B), Janus kinase 3 (JAK3), and signal transducers and activators of transcription 5 (STAT5)], tight junction proteins (claudin 3b, claudin 3c, claudin 11 and occludin), and mitogen-activated protein kinases p38 (p38^{MAPK}) in proximal and distal intestine of juvenile Jian carp after *A. hydrophila* challenge. In contrast, choline deficiency upregulated mRNA levels of antimicrobial peptides (LEAP-2A, LEAP-2B, hepcidin and defensin), cytokines (IL-6b, IFN- γ 2b and TGF- β 2), immune-related signaling molecules (TLR4, MyD88, NF- κ B, I κ B, JAK3, STAT4 in three intestinal segments, and STAT6), claudin 11, and p38^{MAPK} in mid intestine of fish. This study provides new finding that choline deficiency-induced immune responses against *A. hydrophila*

44 infection were varied among three intestinal segments in fish.

45 **Keywords:** Choline, Intestinal inflammation, Jian carp, NF- κ B, Tight junction protein

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1. Introduction

In recent years, the explosive and intensive growth of aquaculture increased the stress on fish, which resulted in the increase of susceptibility to various diseases of fish [1]. Bacterial enteritis has become the most common disease and causes high mortality in fish [2, 3]. Studies showed that *Aeromonas hydrophila* is generally considered as a major pathogen that causes bacterial enteritis in fish, especially freshwater fish [4, 5]. However, treatments of infectious diseases using antibiotics and chemotherapy in fish are limited by the adverse impacts of these treatments, including chemical residues in fish and emergence of drug-resistant bacteria [6]. Nutrient supplementations in fish diets have been an economically promising method to increase disease resistance of fish thus preventing the diseases outbreaks [7-10]. Choline is an essential vitamin for fish [11]. Our previous studies found that choline enhanced the disease resistance and systemic immune response of juvenile Jian carp (*Cyprinus carpio* var. Jian) against *A. hydrophila* infection [12], and the optimal dietary choline requirements for growth, intestinal antioxidant ability and disease resistance of juvenile Jian carp were 566, 884 and 656 mg/kg diet [12-14]. However, whether choline could attenuate the bacterial enteritis caused by *A. hydrophila* in fish is unclear.

Intestinal mucosal immune represents one of the primary barriers against pathogens and plays an important role in protecting fish from the infection of diseases [7, 15]. Meanwhile, the intestinal physical barrier that is formed by the epithelial cells and tight junction proteins also serves as an important line for preventing the entry of

69 pathogens [16, 17]. Study indicated that mRNA levels of pro-inflammatory cytokine
70 tumor necrosis factor α (TNF- α) in intestine was up-regulated in response to *A.*
71 *hydrophila* challenge in grass carp (*Ctenopharyngodon idella*) [4]. In carp, mRNA
72 levels of tight junction proteins including claudin 1 and claudin 11 in intestine were
73 up-regulated during CyHV-3 infection [18]. However, the effects of choline on the
74 intestinal immune and physical barriers after pathogen infection are unknown. Our
75 previous study indicated that serum lysozyme activity, complement 3 (C3) content,
76 and anti-inflammatory cytokine IL-10 gene expressions in spleen and head kidney of
77 juvenile Jian carp after *A. hydrophila* infection were increased by choline, while the
78 gene expressions of pro-inflammatory cytokine TNF- α was downregulated by choline
79 [12]. Furthermore, nuclear factor kappa B (NF- κ B) and signal transducers and
80 activators of transcription (STAT) are two key transcription factors for regulating
81 cytokines transcription [19-21]. Study showed that *A. hydrophila* infection changed
82 gene expressions of NF- κ B p100, inhibitor of NF- κ B (I κ B), STAT1, STAT3 and
83 STAT4 in spleen of common carp [22]. However, whether choline could regulate the
84 NF- κ B and STAT signaling pathways in the intestine of fish after *A. hydrophila*
85 infection is unclear. Choline is an agonist of α 7 nicotinic acetylcholine receptor
86 (α 7nAChR) in mice [23]. Li et al. [24] reported that activating α 7nAChR inhibited the
87 LPS-induced increase of myeloid differentiation primary response 88 (MyD88)
88 protein expression in human airway epithelial cells. In mammal, MyD88 is an
89 important upstream signaling molecule of NF- κ B [25]. Moreover, acetylcholine, the
90 important metabolite of choline, activated STAT1 in human bronchial epithelial cells

[26]. These facts suggest that choline might influence the NF- κ B and STAT pathways in the intestine of fish after pathogen infection, which warrants investigation.

It has been known that fish intestine can be subdivided into three segments [27]. Studies showed that the immune cells and immune genes in each intestinal segment are somewhat different. In Atlantic cod (*Gadus morhua* L.) and common carp (*Cyprinus carpio* L.), the distal part of intestine has much higher number of granulocytes and large Ig-positive macrophages than those in the proximal part of intestine [28, 29]. Moreover, the expression of hepcidin in distal intestine was much higher than that in the proximal intestine of Atlantic cod [28]. Thus, the immune responses of different intestinal segments against pathogens might be varied in fish. Furthermore, the metabolism of choline is somewhat different in different intestinal segments. In pigs, choline is absorbed in the upper small intestine [30]. But choline could be converted into trimethylamine in the lower intestine of rat [31]. Accordingly, choline might mediate different immune responses among different intestinal segments, which needs investigation.

This study expands on our previous investigation which showed that choline improved the systemic and intestinal immune function of fish [12, 32]. Here, the main purpose of the present study was to investigate the effect of **choline deficiency** on bacterial enteritis of fish and reveal the immune responses in different intestinal segments against infection **caused by choline deficiency, which might provide partial theoretical evidence for the choline deficiency-induced decrease of fish disease resistance.**

2. Material and methods

2.1 Diets and growth trail

The present study used one animal trial as our previous study [13]. However, two treatments (choline-deficient group and choline-supplemented group) were just used in the present study. The choline-supplemented group was chose based on the choline requirements for growth, intestinal antioxidant ability and disease resistance of juvenile Jian carp, which were determined to be 566, 884 and 656 mg/kg diet respectively according to our previous study [12-14]. In brief, 400 fish with average initial weight 7.94 ± 0.03 g were randomly assigned to two groups with four replicates each after adaption for one month. As presented in Table 1, the basal diet contained 315.3 g crude protein/kg diet, 44.0 g crude lipid/kg diet and 165 mg choline/kg diet, and used as the choline deficient diet. Choline chloride (Sigma, St Louis, MO, USA) was added to the basal diet to provided 607 mg choline/kg diet (choline-supplemented group). The choline concentration in the experimental diets was assayed according to the method described by Venugopal [33]. The experimental diets were fed to each group to satiation in a closed recirculating water system for 65 days. The Guidelines for the Care and Use of Laboratory Animals of Animal Nutritional Institute, Sichuan Agricultural University were strictly followed during the feeding trail.

2.2 Challenge test

In order to investigate the effects of choline on intestinal immune response against *A. hydrophila* infection, an infection trial was conducted after the growth trial. The challenge experiment was conducted as our previous study [12]. In brief, fish were

chose and intraperitoneally injected with a semilethal dosage of *A. hydrophila* (1.6×10^5 CFU/g fish body weight) in triplicate after growth trial. The *A. hydrophila* was friendly provided by Huazhong Agricultural University, China. Fish were fed the same diets as the feeding trial four times daily, and the moribund fish were recorded and removed daily during the 17 days challenge trial.

2.3 Sampling and measurement of intestinal immune parameters

All sampling procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. At the end of challenge trial, all the live fish from each aquarium were anaesthetized by benzocaine bath (50 mg/L), and the intestine was collected as three intestinal segments (proximal intestine, mid intestine and distal intestine) according to Syakuri et al. [18], then stored at $-70\text{ }^{\circ}\text{C}$ until analyzed. The intestine samples were homogenized in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at $6000\text{ }g$ at $4\text{ }^{\circ}\text{C}$ for 20 min, and the supernatant was conserved for immune parameters analysis. Intestine protein content was determined by method of Bradford [34]. Lysozyme and acid phosphatase (ACP) activity was determined according to the methods described by El-Boshy et al. [35] and Barka and Anderson [36], respectively. C3 and IgM contents were assayed as the procedures described by Welker et al. [37] and Takemura [38], respectively.

2.4 RNA isolation and real-time quantitative PCR

Total RNA was isolated from proximal intestine (PI), mid intestine (MI) and distal intestine (DI) using RNAiso Plus (TaKaRa, Dalian, China) followed by DNase I treatment. RNA concentration and integrity were determined by spectrophotometry at

260 and 280 nm, and by electrophoresis on 1% agarose gels. cDNA was synthesized from RNA by using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China), and then used as the template for amplification of target and housekeeping genes with gene specific primers (Supp. Table 1) using SYBR Green (TaKaRa, Dalian, China) in a CFX96™ Real-Time PCR Detection System (Bio-Rad). Melting curve analysis of amplification products was performed at the end of each amplification to confirm that only a single PCR product was amplified and detected. Relative quantification of target gene was normalized to β -actin content in each sample using the $2^{-\Delta\Delta C_t}$ method [39].

2.5 Statistics

Results were presented as means $\pm$ SE. All data were subjected to Student's t test through SPSS 17.0 (SPSS Inc., Chicago, USA).

3. Results

3.1 Intestinal immune parameters and antimicrobial peptides mRNA levels after challenge

The results of lysozyme, ACP, C3 and IgM are shown in Table 2. Intestinal lysozyme activity, C3 and IgM contents were significantly higher in 607 mg choline/kg diet group than those in the choline-deficient group ($P < 0.05$). However, the intestinal ACP activity was significantly lower in 607 mg choline/kg diet group than that in the choline-deficient group ($P < 0.05$).

The changes of expression levels of LEAP-2A, LEAP-2B, hepcidin and defensin3 genes are showed in Table 3 and Fig. 1. Compared with the choline-deficient group,

the mRNA levels of LEAP-2A, LEAP-2B, hepcidin and defensin-3 in proximal intestine (PI) and distal intestine (DI) were significantly higher in group fed the diet supplemented with 607 mg choline/kg diet ($P<0.05$). However, the mRNA levels of LEAP-2A, LEAP-2B, hepcidin and defensin-3 genes in the mid intestine (MI) were significantly lower in the group fed with choline of 607 mg/kg diet compared with those in the choline deficiency group ($P<0.01$).

3.2 Intestinal cytokine mRNA levels after challenge

As show in Table 3 and Fig. 1, mRNA levels of pro-inflammatory cytokines (IL-6a, IL-6b, TNF- α and IFN- γ 2b) and anti-inflammatory cytokines (IL-10 and TGF- β 2) in three intestinal segments after infection were significantly affected by choline. Compared with the choline-deficient group, IL-6a and TNF- α mRNA levels were significantly upregulated in the PI and DI by choline of 607 mg/kg diet ($P<0.01$) but showed no significant difference in the MI between groups ($P>0.05$). Diet supplemented with 607 mg choline/kg diet upregulated IL-6b mRNA level in the PI ($P<0.05$) while downregulated IL-6b mRNA level in the MI ($P<0.01$) with no significant difference in the DI ($P>0.05$). IFN- γ 2b mRNA levels in the PI and DI were significantly upregulated by choline of 607 mg/kg diet ($P<0.01$), while the mRNA level of IFN- γ 2b in the MI was significantly downregulated by 607 mg choline/kg diet ($P<0.01$). IL-10 mRNA levels in the MI and DI were significantly upregulated by 607 mg choline/kg diet ($P<0.01$), whereas IL-10 mRNA levels in the PI showed no significant difference between groups ($P>0.05$). Compared with the choline-deficient group, diet supplemented with 607 mg choline/kg diet significantly upregulated

mRNA level of TGF- β 2 in the PI ($P<0.05$), but downregulated that in the MI ($P<0.01$) and did not significantly affect that in the DI ($P>0.05$).

3.3 NF- κ B, STAT and related signaling molecules mRNA levels in the intestine after challenge

The **changed** mRNA levels of signaling molecules TLR4, MyD88, NF- κ B and I κ B in the PI, MI and DI are presented in **Table 3 and Fig. 1**. Compared with the choline-deficient group, mRNA levels of TLR4, MyD88, NF- κ B and I κ B in the PI and DI were significantly higher in the group fed with 607 mg choline/kg diet ($P<0.01$), but these mRNA levels in the MI were significantly lower in the group with choline of 607 mg/kg diet ($P<0.01$).

The **changed** mRNA levels of signaling molecules JAK3, TYK2, STAT4, STAT5 and STAT6 in the PI, MI and DI are showed in **Table 3 and Fig. 1**. Compared with the choline-deficient diet, diet supplemented with choline of 607 mg/kg significantly increased JAK3 mRNA levels in the PI and DI ($P<0.05$) but decreased that in the MI ($P<0.01$). Meanwhile, dietary choline (607 mg/kg diet) significantly decreased PI TYK2 mRNA levels ($P<0.01$), while increased MI TYK2 mRNA levels ($P<0.01$) and had no significantly effect on DI TYK2 mRNA levels ($P>0.05$). The mRNA levels of STAT4 in the PI, MI and DI were downregulated significantly by 607 mg choline/kg diet ($P<0.05$). The mRNA levels of STAT5 in the PI and DI were upregulated by 607 mg choline/kg diet ($P<0.01$), while the mRNA levels of STAT5 in the MI showed no significant difference between groups ($P>0.05$). The STAT6 mRNA levels were decreased by 607 mg choline/kg diet in the MI ($P<0.01$), and showed no significant

difference between groups in the PI and DI ($P>0.05$).

3.4 Intestinal tight junction protein mRNA levels after challenge

As showed in Table 3 and Fig. 1, mRNA levels of claudin 3b, claudin 3c and occludin were significantly upregulated by 607 mg choline/kg diet in the PI ($P<0.05$) and DI ($P<0.01$) but showed no significant difference between groups in the MI ($P>0.05$). Compared with the choline deficiency group, diet containing 607 mg choline/kg diet significantly upregulated MI claudin 7 mRNA level ($P<0.01$), but significantly downregulated DI claudin 7 mRNA level ($P<0.01$), and had no significant effect on PI claudin 7 mRNA level ($P>0.05$). Meanwhile, diet containing 607 mg choline/kg diet significantly upregulated claudin 11 mRNA levels in PI and DI ($P<0.01$), but significantly downregulated MI claudin 11 mRNA level ($P<0.01$). The mRNA levels of p38^{MAPK} gene in the PI, MI and DI showed the same patterns as those of the claudin 11 mRNA levels.

4. Discussion

4.1 Choline deficiency depressed the intestinal immune response against *A. hydrophila*

A. hydrophila-induced enteritis is a serious problem for aquaculture. However, nutrient supplementations have been an economically method to increase disease resistance of fish thus preventing the diseases outbreaks [7]. Our previous study reported that dietary choline increased the systemic immunity and resistance against *A. hydrophila* of juvenile Jian carp which was indicated by the decreased mortality in choline-supplemented groups [13]. The mortality after *A. hydrophila* challenge was

53.3% and 20.0% in the choline-deficient group and group with choline of 607 mg/kg diet respectively [12]. In the present study, we investigated the effects of choline on intestinal immune responses after *A. hydrophila* challenge.

Lysozyme and complement factors are useful immune parameters to evaluate the health status of fish [35]. In the present study, dietary choline deficiency decreased lysozyme activity and complement 3 content, as well as IgM content in the intestine of juvenile Jian carp challenged by *A. hydrophila*, suggesting that choline deficiency depressed the intestinal immune responses against *A. hydrophila* infection. Recent study showed that pathogen infection decreased lysozyme C gene expression in intestine of turbot (*Scophthalmus maximus*) [40]. However, soybean oil upregulated intestinal C3 and C4a gene expressions in Senegalese sole (*Solea senegalensis*) [41]. Meanwhile, antimicrobial peptides (AMPs) play an important role in defending microbial invasion in fish [42]. In this study, choline deficiency downregulated LEAP-2A, LEAP-2B, hepcidin and defensin mRNA levels in proximal intestine and distal intestine, indicating that choline deficiency decreased antimicrobial ability in these intestinal segments. However, choline deficiency increased intestinal acid phosphatase activity in the present study. In *Trachinotus ovatus* and *Macrobrachium rosenbergii*, acid phosphatase activity was enhanced after pathogen infection [43, 44]. Thus, the decreased intestinal acid phosphatase activity in choline-supplemented group might indicate that choline attenuated the intestine disease after *A. hydrophila* infection.

Inflammation is an important immune response against bacterial infection and

mediated by cytokines that secreted by activated immune-related cells. Cytokines could be divided into pro-inflammatory cytokines, such as TNF- α and IL-6, and anti-inflammatory cytokines, such as IL-10 and TGF- β . The present study showed that choline supplemented diet upregulated IL-6a, TNF- α , IFN- γ 2b mRNA levels in proximal intestine and distal intestine, IL-6b and TGF- β 2 in proximal intestine, and IL-10 in distal intestine of juvenile Jian carp after challenged with *A. hydrophila*. Recent study suggest that when fish physiology is altered through functional feeds, the host becomes energy efficient rather than immunologically responsive, thereby acquiring the ability to mount a stronger immune response upon a subsequent pathogen encounter [45]. In Jian carp, VD increased the IL-10 mRNA levels in intestine after exposed to lipopolysaccharide [46]. Furthermore, the probiotic *Pediococcus pentosaceus* upregulated the mRNA levels of TNF-2 and TGF- β 1 in head kidney of orange-spotted grouper (*Epinephelus coioides*) after infected with *Vibrio anguillarum* [47]. A mixed-species probiotic also upregulated gene expressions of intestinal TNF- α , IL-1 β , TGF- β and IL-10 in tilapia (*Oreochromis niloticus*) [48]. Soybean oil upregulated intestinal IL-1b and IL-10 gene expressions in Senegalese sole [41]. Accordingly, the upregulated cytokines in the proximal intestine and distal intestine of juvenile Jian carp by choline might help the host to mount an enhanced immune response when challenged with *A. hydrophila*.

NF- κ B is a key transcript factor for regulating transcription of cytokines, and its nuclear translocation can be inhibited by the inhibitory protein I κ B [49]. TLR4 is an important receptor involving in recognizing a variety of highly conserved

pathogen-associated molecular patterns, and can regulate the activation of NF- κ B via the adaptor protein MyD88 in zebrafish [50]. In the present study, choline supplement upregulated mRNA levels of TLR4, MyD88, I κ B and NF- κ B in proximal intestine and distal intestine of juvenile Jian carp after challenged with *A. hydrophila*. Similarly, a mixed probiotic and prebiotic upregulated the gene expressions of TLR3 and I κ B α in intestine of rainbow trout (*Oncorhynchus mykiss*) [51]. Recent study showed that transcription levels of TLR4 and NF- κ B were increased in spleen of common carp at the early time of *Aeromonas hydrophila* infection, however, with the increase of infection time, the enhanced fold of TLR4 transcription levels were decreased, while the transcription levels of NF- κ B decreased [22]. Thus, the immune response of fish against pathogen might increase at the early time of infection while decreased at the later stage. The present data probably indicated that choline could increase the host immune response against pathogen. The results of cytokines in proximal intestine and distal intestine further supported this point. However, the detailed underlying mechanisms need further study.

Meanwhile, STATs is another class of transcription factors of importance for inflammation especial for intestinal inflammation in mammal, and can be activated by the up-stream signaling molecule Janus kinases (i.e. JAK1, JAK2, JAK3 and TYK2) [52]. The current results showed that choline deficiency downregulated mRNA levels of JAK3 and STAT5 in proximal intestine and distal intestine, while upregulated TYK2 mRNA level in proximal intestine, and STAT4 in three intestinal segments of juvenile Jian carp after challenged with *A. hydrophila*. In terrestrial animal,

inflammatory responses were mediated by effector T cells, and an imbalanced effector T cells is a predominant characterization of intestinal inflammatory disease [52]. Naïve CD⁴⁺ T cells can also differentiate into Treg cells, which are characterized by expression of the forkhead box protein 3 (Foxp3) transcription factor and play a central role in establishing and maintaining immunological homeostasis [53]. In mice, complete deletion of STAT5 resulted in dramatic reduction of Treg cells, and JAK3-deficient reduced amounts of T cells as well as low expression of Foxp3 [54]. Furthermore, overexpression of STAT4 exacerbated colitis in mice [55]. Thus, the present data might indicate that choline deficiency impaired intestinal immunological homeostasis and exacerbated intestinal inflammation of juvenile Jian carp after *A. hydrophila* infection.

Besides, the intestinal physical barrier is another important line for preventing the entry of pathogens [16]. Tight junction proteins including occludins and claudins are important components in forming intestinal physical barrier [56]. In terrestrial animal, disappearance of **claudin 3** resulted in the decrease in barrier properties of human intestinal epithelial cell [57]. Claudin 11 was also recognized as a builder of the barrier [18]. Overexpression of claudin 7 decreased the paracellular Cl⁻ conductance and increases the paracellular Na⁺ conductance in porcine kidney epithelial cell line [58]. In mice, knock out of **occludin** induced chronic inflammation of the gastric epithelium [59]. In the present study, choline deficiency downregulated mRNA levels of claudin 3b, claudin 3c, claudin 11 and occludin in proximal intestine and distal intestine, while upregulated claudin 7 mRNA level in the distal intestine of juvenile

Jian carp after challenged with *A. hydrophila*, indicating that choline deficiency impaired the intestinal physical barrier of fish after pathogen infection. In gilthead sea bream (*Sparus aurata* L.), essential oil and prebiotic downregulated gene expressions of claudin 12 and claudin 15, two pore-forming tight junction proteins, in posterior intestine [60]. Furthermore, inhibition of p38MAPK increased occludin expression and prevented the disruption of TJ in human gastric epithelial cells [61]. The present result showed that choline deficiency downregulated mRNA levels of P38^{MAPK} in proximal intestine and distal intestine of juvenile Jian carp after *A. hydrophila* infection. This is in agreement with the results of our previous study, which showed that choline deficiency downregulated intestinal P38^{MAPK} mRNA levels of juvenile Jian carp as an adaptive mechanism [32]. A recent study showed that a mixed-probiotic and prebiotic diet enhanced gene expressions of MAPK1/3 in intestine of rainbow trout [51].

4.2 Choline mediated varied immune responses against *A. hydrophila* in different intestinal segments

In the present study, we subdivided the intestine into proximal intestine, mid intestine and distal intestine according to Syakuri et al. [18], and measured the immune responses in three intestinal segments. Interestingly, we found that choline deficiency-induced immune responses against pathogen infection in proximal intestine and distal intestine were similar, but different from that in mid intestine. For example, choline deficiency downregulated antimicrobial peptides (LEAP-2A, LEAP-2B, hepcidin and defensin), cytokines (IL-6a, TNF- α and IFN- γ 2b), tight

junction proteins (claudin 3b, claudin 3c, claudin 11 and occludin), and related signaling molecules (TLR4, MyD88, NF- κ B, I κ B, JAK3, STAT5 and P38^{MAPK}) mRNA levels in proximal intestine and distal intestine, while upregulated or did not affected **these genes** in mid intestine. However, there is no more information about the relationship between choline and intestinal immunity in vertebrates. The interesting results might be partially explained by the assumption that choline is vital in maintaining normal function of organs or tissues which have much more important functions. In grass carp, the absorptive cell amounts reached the maximum at 20% of gut length and decreased with the gut length, while the pinocytosis of macromolecule took place at posterior intestine [62]. Moreover, many large resident intraepithelial macrophages can be found in the posterior intestine of most cyprinids [27]. These data suggested that the proximal intestine of cyprinids is the major site for nutrients absorption while the posterior intestine in particular is immunologically active among three intestinal segments. Besides, choline is absorbed in the upper small intestine of pigs [30] and could be metabolized into trimethylamine in the lower intestine of rat [31]. Accordingly, choline might play much more important roles in maintaining functions of proximal and distal intestine of fish under disease condition. In terrestrial animal, the liver is central to the degradation of metabolic products [63] and probably the most active organ for choline metabolism [64]. In rat, the liver is the first organ that affected by choline deficiency [65]. This information might support our explanation.

Furthermore, the choline-induced intestinal immune responses after pathogen

infection were also somewhat different from our previous study which found similar immune responses in three intestine segments under choline deficiency without pathogen infection [32]. Similarly, fatty acids-induced immune responses of *Labeo rohita* juveniles were also somewhat different before and after *A. hydrophila* challenge: white blood cells counts and haemoglobin levels were increased before infection, but showed no significant differences among groups after challenge [66]. Yet little is known as to how the immune system is regulated by choline before and after pathogen infection. In terrestrial animal, acetylcholine (ACh) which can be synthesized by choline acetyltransferase (ChAT) from choline and acetyl coenzyme A, plays an important role in regulating inflammation via both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) [67]. Studies showed that ChAT, mAChRs and nAChRs have been identified in various immune cells, including rat and human lymphocytes, as well as human leukemic cell lines [68, 69]. In rat and human, stimulation of T- and B-lymphocytes led to upregulation of ChAT mRNA expression and activity, ACh synthesis and M₅ mAChR expression [70, 71]. Thus, this immunological stimulation-enhanced cholinergic signal transmission might be partly contributed to the difference between immune responses of pre- and post-infection. However, the detailed mechanisms warrant further investigation.

5. Conclusions

The present study showed that dietary choline deficiency exacerbated intestinal inflammation of juvenile Jian carp after *A. hydrophila* challenge, mainly via decreasing intestinal lysozyme activity, C3 and IgM contents, and downregulating

mRNA levels of antimicrobial peptides (LEAP-2A, LEAP-2B, hepcidin and defensin), cytokines (IL-6a, TNF- α , IFN- γ 2b, TGF- β 2 only in proximal intestine and IL-10 only in distal intestine), tight junction proteins (claudin 3b, claudin 3c, claudin 11 and occludin) in proximal and distal intestine. Furthermore, the changes of cytokines mRNA levels might be partly due to the alterations of transcription levels of signaling molecules TLR4, MyD88, NF- κ B, JAKs and STATs induced by choline deficiency, while the changes of TJ proteins transcript abundance might be related to choline-regulated p38^{MAPK} gene expression. However, this study provided two novel findings for understanding the effect of choline on intestinal immunity in vertebrates. First, this study firstly indicated that choline deficiency induced varied immune responses among three intestinal segments of fish after pathogen infection. Second, this study firstly showed that choline deficiency induced different intestinal immune responses of fish before and after pathogen infection. However, further investigation is necessary to fully understand the underlying mechanisms of these choline-mediated intestinal immune responses in vertebrate.

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661 **Table 1** Composition and nutrients content of the basal diet.

Ingredients	g/kg diet	Nutrients content ^a	g/kg diet
Fish meal	20.0	Crude protein	315.3
Soybean protein concentrate	170.3	Crude lipid	44.0
Casein	180.3	Crude ash	48.1
Rice protein meal	33.5	Available phosphorus	6.6
Gelatin	38.2	n-3	10.0
DL-methionine (99%)	4.2	n-6	10.0
Thr (98.5%)	3.6	Methionine	11.3
Fish oil	26.8		
Soy bean oil	16.7		
α-starch	140.0		
Corn starch	276.8		
Ca(H ₂ PO ₄) ₂	19.1		
Choline-free vitamin premix ^b	10.0		
Trace mineral premix ^c	10.0		
Choline chloride premix ^d	30.0		
Ethoxyquin (30%)	0.5		
Cellulose	20.0		

662 ^a Crude protein, crude fat, crude ash, methionine and available phosphorus were
663 measured value. n-3 and n-6 contents were calculated according to NRC [11].

664 ^b Per kilogram of choline-free vitamin premix(g/kg): retinyl acetate (500000 IU/g)

665 0.800g, cholecalciferol (500000 IU/g) 0.480g, DL- α -tocopherol acetate (500 g/kg)
666 20.000g, menadione (500 g/kg) 0.200g, cyanocobalamin (100 g/kg) 0.010g, D-biotin
667 (200 g/kg) 0.500g, folic acid (960 g/kg) 0.521g, thiamin nitrate (980 g/kg) 0.104g,
668 ascorhyl acetate (920 g/kg) 7.247g, niacin (980 g/kg) 2.857g, inositol (980 g/kg)
669 52.857g, calcium-D-pantothenate (980 g/kg) 2.511g, riboflavine (800 g/kg) 0.625g,
670 pyridoxine hydrochloride (980 g/kg) 0.755g. All ingredients were diluted with corn
671 starch to 1 kg.

672 ^c Per kilogram of trace mineral premix (g/kg): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (250.0 g/kg Cu) 1.201g,
673 KI (38.0 g/kg I) 2.895g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (318.0 g/kg Mn) 4.089g, NaSeO_3 (10.0 g/kg Se)
674 2.500g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (197.0 g/kg Fe) 69.695g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (225.0 g/kg Zn) 21.640g.
675 All ingredients were diluted with CaCO_3 to 1 kg.

676 ^d Per kilogram of choline chloride premix (g/kg): each treatment group containing
677 choline chloride 0g, 5.5170g, 17.2769g, 29.0368g, 40.7967g and 64.3166g,
678 respectively. Each choline chloride mixture was diluted with corn starch to 1 kg.
679

Table 2 Effects of choline on intestinal activities of lysozyme and acid phosphatase (ACP), contents of complement 3 (C3) and IgM of juvenile Jian carp after infected with *A. hydrophila*.

Choline (mg/kg diet)	165	607
lysozyme (U/mg protein)	38.297±4.880	85.536±5.520 ^{**}
ACP (U/mg protein)	109.869±3.122 ^{**}	94.277±2.427
C3 (g/g protein)	0.443±0.044	0.785±0.091 [*]
IgM (g/g protein)	0.391±0.039	0.948±0.131 [*]

Values are means ± SE (n=6). ^{*} $P < 0.05$, ^{**} $P < 0.01$.

Table 3 Choline deficiency-induced fold-changes of mRNA levels of antimicrobial peptide, cytokines, immune-related signaling molecules, tight junction protein and related signaling molecules in the proximal intestine, mid intestine and distal intestine of juvenile Jian carp after infected with *A. hydrophila*.

Gene name	Proximal intestine	Mid intestine	Distal intestine
<i>Antimicrobial peptides</i>			
<i>Defensin-3</i>	-0.806**	7.507**	-0.565**
<i>Hepcidin</i>	-0.622**	1.772**	-0.540**
<i>Liver-expressed antimicrobial peptide 2A</i>	-0.484**	2.886**	-0.640**
<i>Liver-expressed antimicrobial peptide 2B</i>	-0.333*	2.035**	-0.771**
<i>Cytokines</i>			
<i>Tumor necrosis factor α</i>	-0.531**	-0.066	-0.561**
<i>Transforming growth factor β2</i>	-0.293*	1.164**	-0.213
<i>Interleukin 6a</i>	-0.538**	-0.208	-0.326**
<i>Interleukin 6b</i>	-0.385**	4.054**	0.078
<i>Interleukin 10</i>	-0.189	-0.979**	-0.543**
<i>Interferon γ2b</i>	-0.567**	1.941**	-0.691**
<i>Immune-related signaling molecules</i>			
<i>Inhibitor of NF-κB</i>	-0.714**	2.330**	-0.458**
<i>Janus kinase 3</i>	-0.642**	2.332**	-0.286*
<i>Myeloid differentiation primary response 88</i>	-0.678**	1.762**	-0.702**
<i>Nuclear factor kappa B</i>	-0.626**	2.904**	-0.638**

<i>Signal transducers and activators of transcription 4</i>	0.307*	1.811**	0.272*
<i>Signal transducers and activators of transcription 5</i>	-0.684**	0.419	-0.650**
<i>Signal transducers and activators of transcription 6</i>	-0.166	9.049**	0.052
<i>Toll-like receptor 4</i>	-0.681**	1.616**	-0.558**
<i>Tyrosine kinase 2</i>	1.407**	-0.741**	-0.077
<i>Tight junction protein and related signaling molecules</i>			
<i>Claudin 3b</i>	-0.514**	-0.231	-0.450**
<i>Claudin 3c</i>	-0.567**	-0.294	-0.610**
<i>Claudin 7</i>	-0.081	-0.846**	0.919**
<i>Claudin 11</i>	-0.643**	2.234**	-0.423*
<i>Occludin</i>	-0.331*	-0.225	-0.517**
<i>Mitogen-activated protein kinase p38</i>	-0.743**	3.173**	-0.671**

Bold values indicate that the gene is significantly changed compared to that in the group with choline of 607 mg/kg diet. * $P < 0.05$; ** $P < 0.01$.

Fig. 1 Heat-map of choline-changed expression of selected genes in the proximal intestine, mid intestine and distal intestine of juvenile Jian carp after infected with *A. hydrophila*. The signal values of up-regulation (red) and down-regulation (blue) were expressed as Log2Fold and ranged from 5 to -5 folds.